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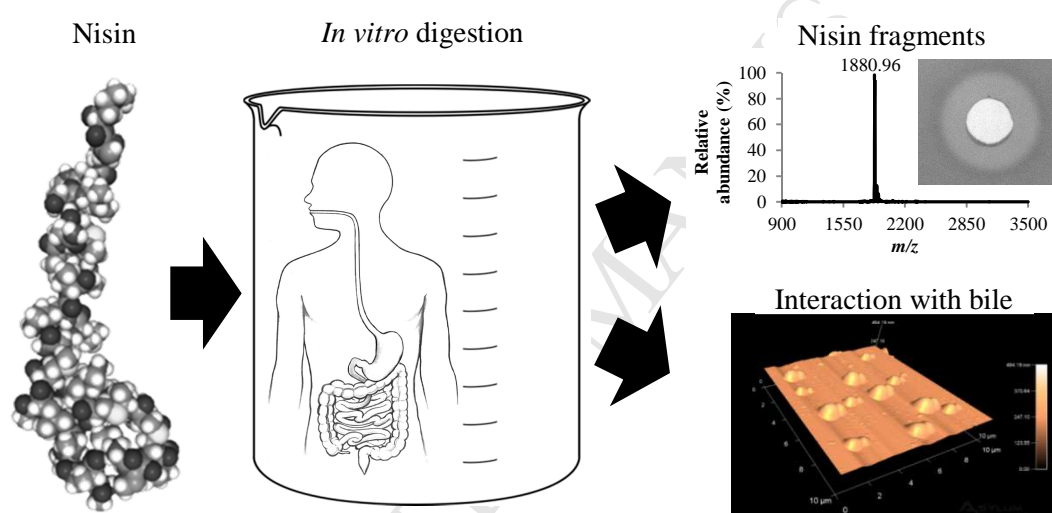
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# Simulated gastrointestinal digestion of nisin and interaction between nisin and bile

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## Abstract

Nisin, an antimicrobial peptide showing activity against many Gram positive bacteria, is widely used as a food preservative. The simulated gastrointestinal digestion of nisin (variant A) was studied using the *in vitro* INFOGEST digestion method. Following oral, gastric and small intestinal digestion, there was no intact nisin in the system and the nisin was primarily digested by pancreatin. After digestion, six nisin fragments (1-11, 1-12, 1-20, 1-21, 1-29 and 1-32) were identified by reversed phase high performance liquid chromatography and mass spectroscopy and four of these nisin fragments (1-20, 1-21, 1-29 and 1-32) demonstrated low antibacterial activity against *Lactococcus lactis* HP in agar diffusion activity assays. Additionally, it was observed that bile salts form a complex with nisin. This was examined by atomic force microscopy, turbidity and dynamic light scattering, which showed that this interaction resulted in significantly larger bile salt micelles. The presence of bile salts at physiological levels significantly altered the relative amounts of the nisin fragments 1-12, 1-20 and 1-29 produced during an *in vitro* digestion. This study highlights the importance of including bile in simulated digestions of antimicrobial peptides in order to obtain a more accurate simulation of the *in vivo* digestion products and their activity.

## Keywords:

Nisin; *In vitro* digestion; Bile; Antimicrobial peptide; Surfactant

## 1. Introduction

Nisin is a 34 amino acid antimicrobial peptide produced by strains of *Lactococcus lactis* subsp. *lactis* that is active against many Gram-positive bacteria and is widely used as a food preservative (Gharsallaoui et al., 2016). Nisin is extremely stable at pH 3 and can be autoclaved at this pH with < 5% loss of activity (Davies et al., 1998), whereas above pH 6 it is unstable even at room temperature (Kelly et al., 2000).

The discovery that nisin is inactivated by pancreatin (Heinemann and Williams, 1966), primarily due to its chymotrypsin component (Jarvis and Mahoney, 1969), was a factor in nisin being awarded GRAS status by the FDA (U. S. Food and Drug Administration, 1988) and the European Food Safety Authority declaring that nisin is safe for use in food (European Food Safety Authority, 2006) with its assigned E number being E 234 (European Commission, 2011). It has been demonstrated more recently that nisin is also cleaved by the trypsin component of pancreatin (Chan et al., 1996). However these studies focused on pancreatic enzymes and did not take into account the other components of the digestive system such as bile.

Bile salts, the major functional component of bile, are biological surfactants which are involved in the digestion and absorption of lipids in the small intestine; in particular they transport the products of lipolysis in bile salt micelles to the sites of absorption (Bauer et al., 2005). For the most common human and porcine bile salts, micelle formation takes place in two stages; hydrophobic interactions between bile salts results in primary micelles, which then interact via hydrogen bonding to form secondary micelles (Kandrac et al., 2006; Partay et al., 2007; Small, 1968). The minimum bile salt concentration required for micelle formation is termed the critical micelle concentration (CMC). As the concentration of sodium ions affects the CMC, experiments with bile salts are commonly performed in 0.15 moles/L Na<sup>+</sup> solutions to simulate physiological conditions (Hofmann and Hagey, 2008). In a 0.15 moles/L Na<sup>+</sup> solution, most bile salts have a CMC below 10 millimoles/L (Hofmann and Roda, 1984); 10 millimoles/L is also the bile salt concentration recommended for simulating physiological conditions during *in vitro* digestion (Minekus et al., 2014).

Previous digestion studies on nisin have focussed on pancreatic enzymes from the small intestine and those that investigated the nisin fragments produced by digestion used enzymes individually and often used digestions in excess of 20 h (Chan et al., 1996; Heinemann and Williams, 1966; Jarvis and Mahoney, 1969; Slootweg et al., 2013). In order to study how nisin is digested under more physiologically relevant conditions, the INFOGEST method, a recently developed standardized static method for the digestion of food (Minekus et al., 2014) was utilised. This method is the consensus of an international network of scientists and is based on

physiological conditions with each digestion comprising an oral, gastric and intestinal stage (Minekus et al., 2014). This approach would establish which nisin fragments are produced under physiological conditions and also their biological activity. In addition, by performing versions of the digestion without individual digestion components, the importance of non-proteolytic components such as bile on the digestion profile of nisin could be established.

## 2. Materials and methods

### 2.1. Materials

All reagents were obtained from Sigma-Aldrich (Arklow, Ireland) unless otherwise stated. For the simulated digestions the specific Sigma-Aldrich products used were: salivary amylase (A1031), pepsin (P6887), bile (B8631) and pancreatin (P7545). Tween<sup>®</sup> 80 was obtained from Merck Millipore (Darmstadt, Germany). The nisin preparation used was Nisaplin<sup>®</sup> (DuPont, Beaminsten, UK) (nisin variant A; referred to as 'nisin' throughout this text). This was enriched by salting out as previously described (Gough et al., in press).

### 2.2. Digestion

Simulated oral, gastric and small intestinal digestions were performed as described in the INFOGEST method (Minekus et al., 2014). Five variations of the digestion were performed: (i) nisin with all digestion components, (ii) nisin with all digestion components except bile, (iii) nisin with all digestion components except pancreatin, (iv) nisin with all digestion components except pepsin, bile and pancreatin, (v) all digestion components but no nisin. A minimum of three replicates were performed of each of these five digestion setups. The initial nisin concentration was chosen so that the nisin concentration in the digestion product would be sufficient for quantification by reversed phase - high performance liquid chromatography (RP-HPLC). The digestion containing nisin and all digestion components was performed as follows: for the oral stage 5 mL of an 8.7 mg/mL nisin solution was combined with simulated salivary fluid (SSF) and salivary amylase (75 U/mL in final oral solution) to a final total volume of 10 mL; this was incubated at 37 °C for 2 minutes. For the gastric stage, the sample pH was adjusted to 3 using dilute HCl and combined with simulated gastric fluid (SGF) and pepsin (2,000 U/mL in final gastric solution) to a final total volume of 20 mL; this was incubated at 37 °C for 2

hours. For the small intestinal stage the pH was adjusted to 7 using dilute NaOH and combined with simulated intestinal fluid (SIF) and bile (10 millimoles/L bile salts in final intestinal solution) and pancreatin (100 TAME U/mL in final intestinal volume) to a final total volume of 40 mL, this was incubated at 37 °C for 2 hours. The digestion products were snap-frozen in liquid nitrogen.

### 2.3. Determination of the effect of the presence of bile during digestion on the activity of the digestion products

To determine the effect of the presence of bile during digestion on the activity of the digestion products a simplified digestion method based on Minekus et al. (2014) was used; nisin was incubated with pancreatin in a MOPS buffer at pH 7 and 37 °C for 2 h with bile added either before or after digestion, with an equivalent volume of water added to samples that did not receive bile. The final constituents in each sample, in a total volume of 0.5 mL, were 100 µg/mL nisin, bile at a bile salt concentration of 0.3 millimoles/L, pancreatin at a concentration such that its trypsin activity was 100 TAME units per mL, 50 millimoles/L MOPS, 0.15 moles/L NaCl and the pH was 7. The digestion products were analysed by activity assay as described in section 2.6.

### 2.4. Reversed Phase - High Performance Liquid Chromatography (RP-HPLC)

RP-HPLC was carried out on a Jupiter, 5 µm, C18, 300 Å, 250 mm × 4.6 mm column from Phenomenex (Macclesfield, UK) with an acetonitrile (Thermo Fisher Scientific, Dublin, Ireland) gradient as described previously (Gough et al., in press). In the case of digested nisin, fractions were collected throughout the gradient to determine the nisin fragments produced by digestion.

### 2.5. Matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI TOF MS)

The molecular mass of the RP-HPLC peaks were determined using MALDI TOF MS using an Axima TOF<sup>2</sup> mass spectrometer (Shimadzu Biotech, Kyoto, Japan) as previously described (Field et al., 2012).

### 2.6. Activity Assay

Biological activity was estimated by agar diffusion activity assays (Ryan et al., 1996) in agar plates seeded with *Lactococcus lactis* subsp. *cremoris* HP which were prepared as described previously (Gough et al., in press). Serial two-fold dilutions of the samples were performed in 0.15 moles/L NaCl, 50 millimoles/L MOPS, pH 7. In specific cases a surfactant (0.3 millimoles/L bile salts, 8 millimoles/L Tween<sup>®</sup> 80 or 0.2 millimoles/L Triton<sup>™</sup> X-100) was included in the diluent. The samples (50 µL) were dispensed into the wells and the plates incubated overnight at 30 °C. The activity of nisin resulted in zones of inhibition surrounding the wells. Activity is expressed as minimum inhibitory concentration (MIC) in terms of µg/mL (Chan et al., 1996). MIC was calculated by plotting the area of the zone of inhibition at each dilution stage against the log of the nisin concentration (Bernbom et al., 2006); these had a linear relationship and the MIC was calculated from the equation of the line.

## 2.7. Atomic Force Microscopy (AFM)

For AFM, samples comprised 10 millimoles/L bile salts, 0.15 moles/L NaCl, and 50 millimoles/L MOPS at pH 7, with or without 0.5 µg/mL nisin. The nisin concentration of 0.5 µg/mL was chosen as this is within the range that could occur in the small intestine after consumption of a nisin containing foodstuff (Delves-Broughton, 2005; Minekus et al., 2014). Aliquots (5 µL) were deposited onto freshly cleaved mica surfaces, dried in a desiccator and subsequently stored at ambient conditions to ensure equilibrated hydration. AFM images were obtained with an Asylum Research MFP-3D-AFM (Asylum Research UK Ltd., Oxford, UK) using AC-mode in ambient air. An aluminium reflex coated cantilever with a tetrahedral tip (AC 240), spring constant of 1.8 N/m (Olympus Optical Co. Ltd., Tokyo Japan), working frequency of 50-90 kHz, and scan rate of 0.5-1 Hz was used at a 512 × 512 resolution. The radius of curvature of the tetrahedral tip was 10 (± 3) nm.

## 2.8. Turbidity

Turbidity was measured at 600 nm as per (Dahmane et al., 2008) using a Cary 100 Bio Spectrophotometer with temperature control (Agilent Technologies Ireland Ltd., Little Island, Ireland). The samples were prepared at pH 7 in a 50 millimoles/L MOPS buffer containing 0.15 moles/L NaCl and analysed at 37 °C.



## 2.9. Dynamic Light Scattering

Z-average was measured by Dynamic Light Scattering (DLS) using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK). The samples were prepared in 0.15 moles/L NaCl, adjusted to pH 7 using NaOH, filtered through a 0.22  $\mu$ M PVDF filter (Gilson Scientific, Luton, UK) and analysed at 20 °C.

## 3. Results and discussion

### 3.1. Simulated digestion

Oral and gastric digestion of nisin without pepsin resulted in a 6% ( $\pm 0.6$ ,  $n = 3$ ) reduction in intact nisin when measured by RP-HPLC and the inclusion of pepsin brought the total reduction to 16% ( $\pm 2.2$ ,  $n = 5$ ); this limited digestion in the oral and gastric stages correlates with published results which show that nisin is primarily digested in the small intestine (Jarvis and Mahoney, 1969).

Gastrointestinal digestion without proteases or bile resulted in  $> 50\%$  loss in intact nisin (Table 1). As the oral and gastric stages caused limited reduction in nisin, this reduction can primarily be attributed to the small intestinal pH of 7 and temperature of 37 °C, as above pH 6 nisin is unstable with a temperature dependent decomposition rate (Kelly et al., 2000). It was noted that pH and temperature were not entirely responsible for the reduction in detectable nisin and that the simulated intestinal fluid, in particular its sodium bicarbonate component, played a minor role (data not shown).

Performing the small intestinal stage of digestion with bile and/or pancreatin resulted in no intact nisin being detectable by RP-HPLC (Table 1) and the products of digestions that included bile had greater antibacterial activity than similar digestions without bile.

The highest antibacterial activity was in digestions without pancreatin; this correlates with previous reports that pancreatin is primarily responsible for nisin digestion (Heinemann and Williams, 1966). However in digestions with all components except pancreatin, there was no intact nisin detected by RP-HPLC. The high antibacterial activity implies that intact nisin was present and suggests another digestion component may be affecting the behaviour of nisin on the RP-HPLC column, thus interfering with its detection. This component appeared to be bile, as digestions without bile or proteases had detectable nisin in their products.

### 3.2. Analyses of nisin fragments

As the products of digestions involving pancreatin demonstrated antibacterial activity that could not be accounted for by the bile or digestive enzymes, the activity was likely due to fragments of nisin. To determine which nisin fragments were produced and which of these were bioactive, the digestion products were separated by RP-HPLC and the fractions collected (Fig. 1B). The fractions were analysed using MALDI-TOF mass spectrometry and by activity assay (Fig. 2). Digestions with pancreatin produced peptides with molecular masses corresponding to the theoretical and published molecular masses of nisin fragments 1-12, 1-20, 1-21, 1-29 and 1-32 and also a peptide with a molecular mass within two daltons of the theoretical mass of nisin fragment 1-11 (Table 2).

Major peaks on the RP-HPLC traces (Fig. 1A and B) corresponded to nisin 1-12, 1-20, 1-29 and intact nisin, whereas nisin 1-21 gave a minor peak. Intact nisin had a shoulder region corresponding to the nisin variant [Ser<sup>33</sup>]-nisin in which the serine residue at position 33 did not undergo post-translational modification to dehydroalanine (Chan et al., 1996). The peaks corresponding to nisin fragments 1-11 and nisin 1-32 were obscured by the co-eluting bile and pancreatic components. Although the RP-HPLC peak of nisin 1-32 was completely obscured by the background, its elution point was identified by activity assay.

Nisin 1-29 eluted at slightly different time points depending on whether the digestion was performed without bile (25.1 min) or with bile included (25.4 min). The peaks at 22.8 min and 26.9 min and the four major peaks between 25.5 min and 26.1 min were primarily due to bile and pancreatin and occurred in the control digestions that did not have nisin. The presence of bile in the digestion affected the peak heights of nisin fragments 1-12, 1-20 and 1-29 (Fig. 1B).

RP-HPLC fractionation did not lead to pure peptide fractions due to overlap between the elution of the fragments, for example in Fig. 2 nisin fragment 1-29 was detected in the mass spectrometry analysis of the elution peak of nisin fragment 1-11 and was most likely the source of the antimicrobial activity in the activity assay of nisin fragment 1-11, also nisin fragment 1-20 was visible in the mass spectrometry analysis of the elution peak of nisin fragment 1-32.

Nisin fragment 1-32 was not detected in the products of digestion in the presence of bile; as bile interfered with the detection of intact nisin (1-34) by RP-HPLC, it is proposed this also occurs with nisin fragment 1-32.

Nisin fragments 1-12, 1-20 and 1-21 have previously been produced by digests with trypsin or chymotrypsin for a minimum of 16 hours (Chan et al., 1996; Slootweg et al., 2013). Nisin fragment 1-29 has been produced by an 8 hour digestion with the bacterial protease thermolysin and by a 4 hour digestion of the nisin variant ([Ser<sup>33</sup>]-nisin) with carboxypeptidase Y (Chan et al., 1996). Nisin fragment 1-32 has been produced by a 6 day acid treatment (Chan et al., 1989; Chan et al., 1996). To the authors knowledge it has not been previously demonstrated that these fragments can be produced under physiological conditions.

In Table 2 it is shown that the molecular masses observed in Fig. 2 are within two daltons of the predicted masses and that the inclusion of bile in a digestion altered the proportions of the nisin fragments produced when compared by peak height in a RP-HPLC chromatogram. In this study all the nisin fragments corresponding to amino acids 1-20 or longer demonstrated antibacterial activity against *Lactococcus lactis* (Fig. 2), which is in agreement with Chan et al. (1996). The decrease in nisin 1-12 and an increase in nisin 1-20 and 1-29, which are observed in Fig. 1B are shown to be significant (Table 2). This implies that the bile reduces the cleavage of nisin during digestion.

### 3.3. Nisin interaction with bile and other surfactants

As bile had been shown to increase nisin activity (Table 1) and alter its digestion (Table 2) the bile-nisin interaction was further examined and compared to nisin's interaction with other surfactants. The surfactants Tween<sup>®</sup> 80 and Triton<sup>™</sup> X-114 were chosen for comparison as they increase the activity of nisin (Joosten and Nunez, 1995; Jozala et al., 2008). Triton<sup>™</sup> X-100 was substituted for Triton<sup>™</sup> X-114, as Triton<sup>™</sup> X-114 phase separates at the incubation temperature of the activity assay (30 °C) (Bordier, 1981). Regarding the concentrations used; 10 millimoles/L bile salts is physiological concentration (Minekus et al., 2014), while 0.3 millimoles/L bile salts and 0.2 millimoles/L Triton<sup>™</sup> X-100 were the highest concentrations that did not cause antibacterial activity in activity assays. While Tween<sup>®</sup> 80 did not have an antibacterial affect at concentrations > 8 millimoles/L, 8 millimoles/L was chosen because higher concentrations had a noticeable effect on viscosity. All these surfactants were at a molar excess over the nisin component (100 µg/mL nisin ≈ 0.03 millimoles/L nisin).

To investigate how surfactants affected the MIC of nisin when determined by activity assays; serial dilutions were performed in a MOPS/NaCl diluent on its own and with each of the surfactants, with a starting nisin concentration of 100 µg/mL (Table 3). Bile caused a reduction in MIC compared to the control, however a

greater reduction was caused by Tween<sup>®</sup> 80 and Triton<sup>™</sup> X-100 and both of these reduced the MIC by the same amount (Table 3). This implies that nisin interacts differently with bile compared to the other surfactants.

Surfactants can increase the activity of bioactive peptides in activity assays by reducing or preventing binding to glass or polypropylene assay containers through competition with the peptides for binding sites on the container or interacting with the binding sites on the peptides, primarily through hydrophobic interactions (Duncan et al., 1995; Joosten and Nunez, 1995). If nisin activity was increased by bile, through the formation of a peptide-surfactant complex, there would also be an increase in the particle size of the bile salt micelle.

The effect of nisin-surfactant interaction on particle size was examined by AFM, turbidity and DLS. AFM analysis of bile with and without nisin (Fig. 3A and B) showed individual particles whose cross-sections had z-heights ranging from 20 to 190 nm, which was similar to the z-average means obtained by DLS for bile (100 nm  $\pm$  5) and bile with nisin (118 nm  $\pm$  9) (Fig. 3D) and similar to the published values for bile salt secondary micelles (50 to 200 nm) (Hildebrand et al., 2004).

The turbidity of a system relates to both the size and density of particles that scatter light. In Fig. 3C, nisin was solubilised in a range of surfactants and turbidity increased with increasing nisin concentration, with the greatest turbidity increases occurring in the presence of bile. DLS (Fig. 3D) found that the z-average diameter of 10 millimoles/L bile salts with 100  $\mu$ g/mL nisin was significantly larger than that without nisin ( $p = 0.0123$ ,  $n = 4$ ). There was no significant change in particle size when nisin was in solution with Triton<sup>™</sup> X-100 or Tween<sup>®</sup> 80 (data not shown). To produce results of suitable quality by DLS, the samples were filtered and measured at 20 °C and pH 7 without a buffer.

The particle size analysis (Fig. 3) suggests that nisin formed a complex with bile and this was different to its interaction with other surfactants. In the activity analysis (Table 3) bile caused less of an increase in nisin activity than the other surfactants; this could be explained by bile forming a complex with nisin whereas the other surfactants bound to the assay container and thus reduced non-specific nisin binding in a different way. In the products of digestions which contained all digestion components except pancreatin (Table 1), nisin was detected by activity assay but not detected by RP-HPLC; nisin could be favouring hydrophobic interaction with bile over hydrophobic interactions with the RP-HPLC column.

With respect to the mechanism for bile-nisin interaction, both hydrophobic and ionic interactions are possible. Bile salts are anionic with a negative charge on an amino acid that is attached to one end of the main body of the bile salt via a short hydrocarbon chain (Fig. 4) (Hofmann and Hagey, 2008; Small, 1968). Nisin has a pI of 8.5 and thus is cationic under physiological conditions with the bulk of the positive charge being in the

C-terminal domain (Fig. 4) and this is responsible for its initial interaction with its negatively charged targets (Breukink et al., 1997). The oppositely charged terminal regions of bile salts and nisin make ionic interactions likely. Bile salts are planar amphipathic molecules and thus have a hydrophobic and a hydrophilic side (Fig. 4); when forming a primary micelle they orientate their hydrophobic sides towards each other, giving the micelle a hydrophobic core (Fig. 4) (Hofmann and Hagey, 2008). Nisin is also amphipathic with the C-terminal being hydrophilic while the N-terminal is hydrophobic (Fig. 4) (Gharsallaoui et al., 2016). The hydrophobic N-terminal region of nisin has been reported to interact with the hydrophobic cores of surfactants such as dodecylphosphocholine and sodium dodecyl sulphate micelles (van den Hooven et al., 1996). It is therefore possible that the hydrophobic N-terminal region of nisin also interacts with the hydrophobic core of bile salt micelles. The reduced cleavage in the N-terminal region of nisin when a simulated gastrointestinal digestion is performed in the presence of bile (Table 2) may be due to the bile salts interacting with and surrounding the N-terminal region of the nisin and limiting the capacity of proteolytic enzymes to interact with the N-terminal region.

Having shown that nisin formed a complex with bile salts, which altered its digestion products, it was investigated how much this affected its antibacterial activity after digestion. The loss of nisin activity during digestion is primarily due to pancreatic enzymes (Section 3.1). To look specifically at whether bile could attenuate the loss of nisin activity due to pancreatin, a simplified digestion was performed with pancreatin in buffer and bile added at the beginning or end of the digestion and the activity of the product determined by activity assay. The final concentration of bile salts was 0.3 millimoles/L so that the antibacterial activity of bile salts would not distort the results of the activity assay. The MOPS/NaCl buffer (pH 7) that was used as a diluent for the activity assay contained 0.3 millimoles/L bile salts, so that the surfactant effect would be consistent at all stages of the assay. The addition of bile before or after a 2 h digestion resulted in MICs of 11.8 µg/mL ( $\pm$  0.3,  $n$  = 3) and 12.7 µg/mL ( $\pm$  0.2,  $n$  = 3) respectively. Although statistically significant ( $p$  = 0.01), the difference in activity was slight.

Although the presences or absence of bile in a static *in vitro* digestion had a significant effect on the nisin fragments produced, this in turn had a negligible effect on antibacterial activity. As nisin fragments 1-12, 1-20 and 1-29 have low antibacterial activity (< 6% the activity of intact nisin against *L. lactis* (Chan et al., 1996)) it is assumed that increases or decreases in their amount had a minimal effect on overall antibacterial activity.

#### 4. Conclusions

Nisin was digested by intestinal proteases as has been previously highlighted (Heinemann and Williams, 1966; Jarvis and Mahoney, 1969) although intestinal pH and temperature by themselves also caused significant loss in nisin. The digestion products include six nisin fragments, four of which have limited antibacterial activity. Although nisin fragments have been previously identified (Chan et al., 1996; Slootweg et al., 2013), this is the first time that fragments generated during an *in vitro* digestion under physiological conditions have been identified. Nisin interacts with bile, forming a complex that alters the relative amounts of the nisin fragments produced by digestion. This study highlights the importance of including bile in simulated digestions of antimicrobial peptides regardless of the presence or absence of a lipid component in the test samples.

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**Table 1**Products of *in vitro* gastrointestinal digestions of nisin.

Starting material	Oral, gastric and small intestinal digestion	Analysis of digestion products	
		% nisin (RP-HPLC)	MIC (or MIC equivalent)
Nisin solution	All components	0% ( $\pm$ 0)	22 $\mu$ g/mL ( $\pm$ 5)
Nisin solution	All components except bile	0% ( $\pm$ 0)	41 $\mu$ g/mL ( $\pm$ 2)
Nisin solution	All components except pancreatin	0% ( $\pm$ 0)	0.9 $\mu$ g/mL ( $\pm$ 0.2)
Nisin solution	All components except pepsin, bile and pancreatin	48% ( $\pm$ 2)	2.9 $\mu$ g/mL ( $\pm$ 0.5)
H <sub>2</sub> O (no nisin)	All components	n/a	82 $\mu$ g/mL ( $\pm$ 21)
Nisin solution	Not digested	100%	1.8 $\mu$ g/mL ( $\pm$ 0.1)

The digestion products were analysed by RP-HPLC and agar diffusion activity assay. The amount of nisin detected by RP-HPLC is expressed as a % of the total initial nisin. Activity is expressed as minimum inhibitory concentration (MIC,  $\mu$ g/mL). An equivalent MIC is given for the products of digestions without nisin; SD in brackets,  $n \geq 3$ .

**Table 2**

Nisin fragments detected in the products of digestion.

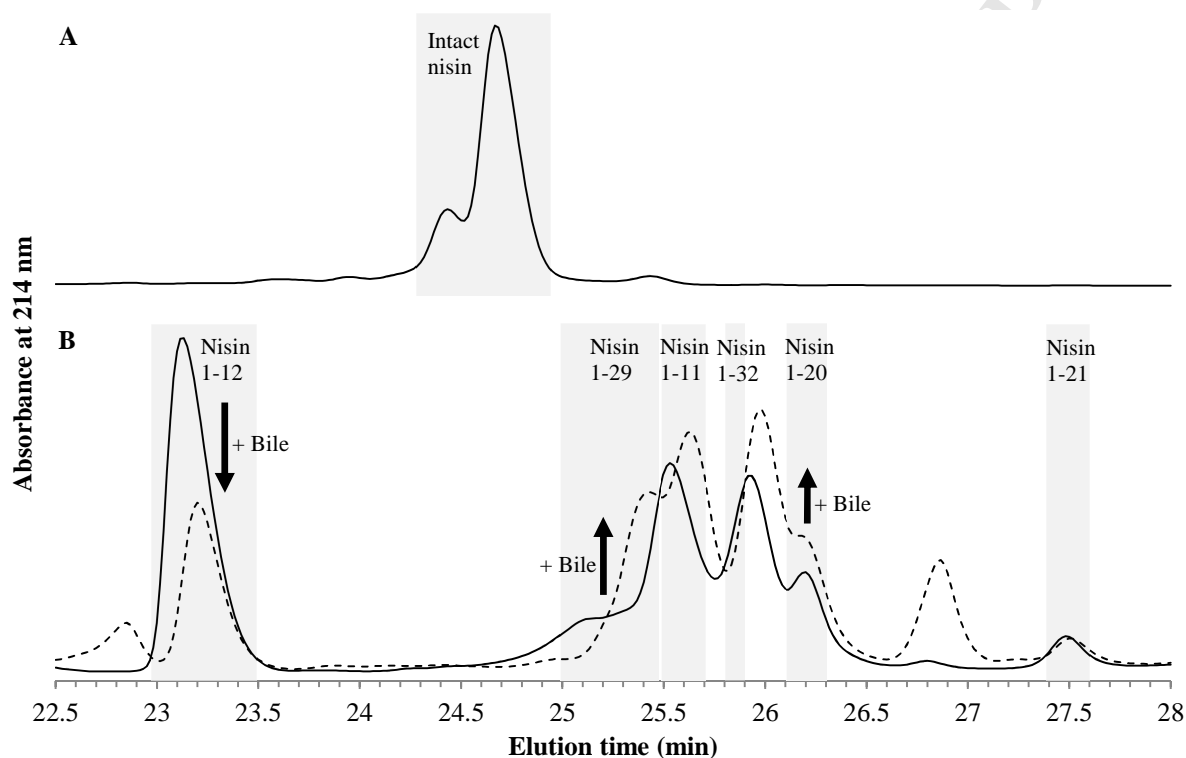
Nisin fragment	Observed / predicted molecular mass	Antibacterial activity	Effect of inclusion of bile in digestion on height of corresponding peak in RP-HPLC chromatogram
1-11	1023 / 1021	None detected	Peak height not determinable due to background interference from co-eluting bile and pancreatin
1-12	1151 / 1150 <sup>b</sup>	None detected	1.9 ( $\pm$ 0.3) fold decrease ( $p = 0.0009$ )
1-20	1881 / 1881 <sup>a</sup>	Yes	1.4 ( $\pm$ 0.1) fold increase ( $p = 0.02$ )
1-21	2013 / 2012 <sup>b</sup>	Yes	Not significant ( $p = 0.06$ )
1-29	2810 / 2809 <sup>a</sup>	Yes	3.5 ( $\pm$ 0.3) fold increase ( $p < 0.0001$ )
1-32	3159 / 3157 <sup>a</sup>	Yes	Peak height not determinable due to background interference from co-eluting pancreatin

Mean fold increases and SD are derived from three sets of replicates;  $p$  values are in brackets.<sup>a</sup>Chan et al. (1996).<sup>b</sup>Slotweg et al. (2013).

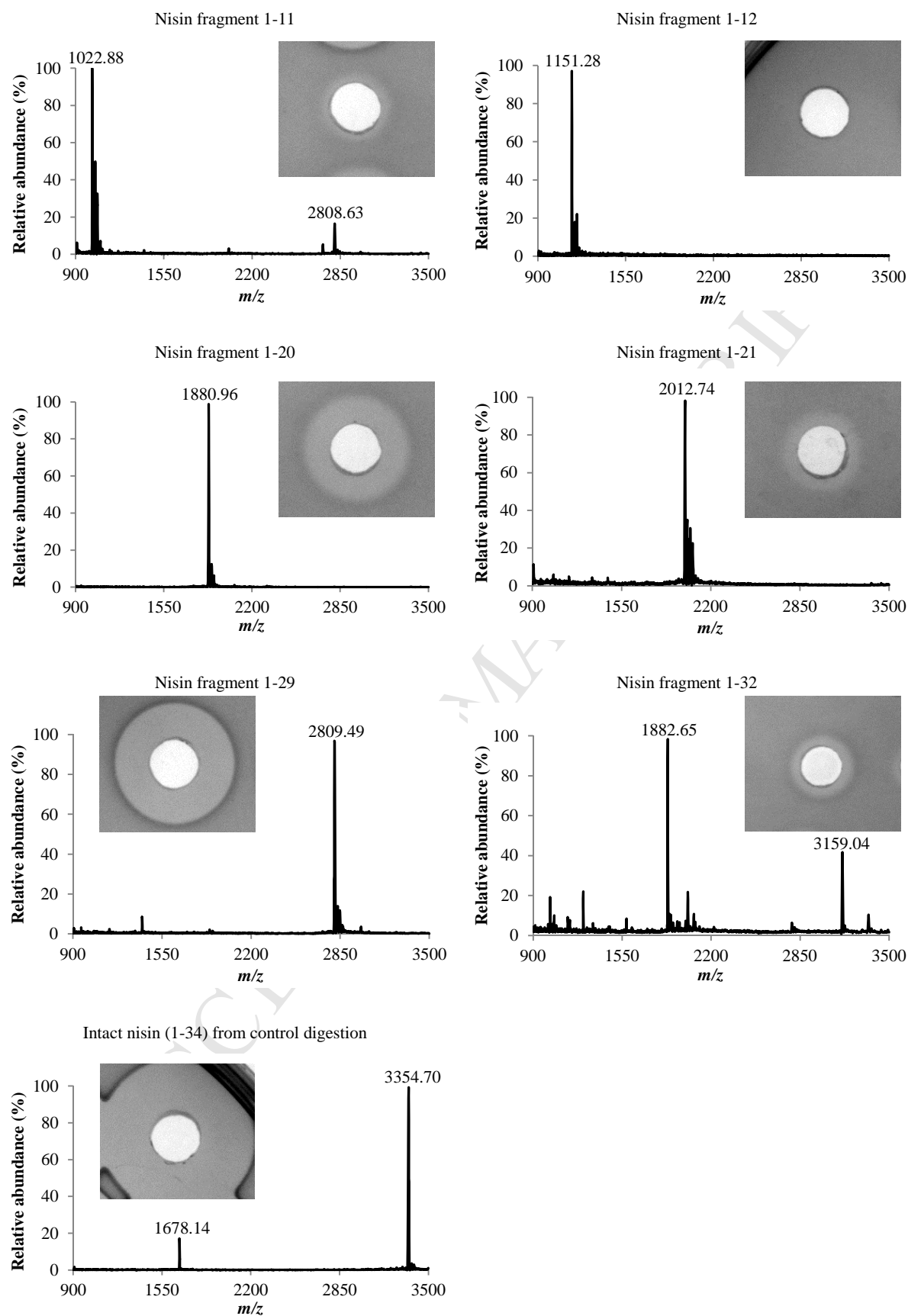
**Table 3**

Effect of surfactants on the minimum inhibitory concentration (MIC,  $\mu\text{g/mL}$ ) of nisin in agar diffusion activity assays (SD in brackets,  $n=3$ ).

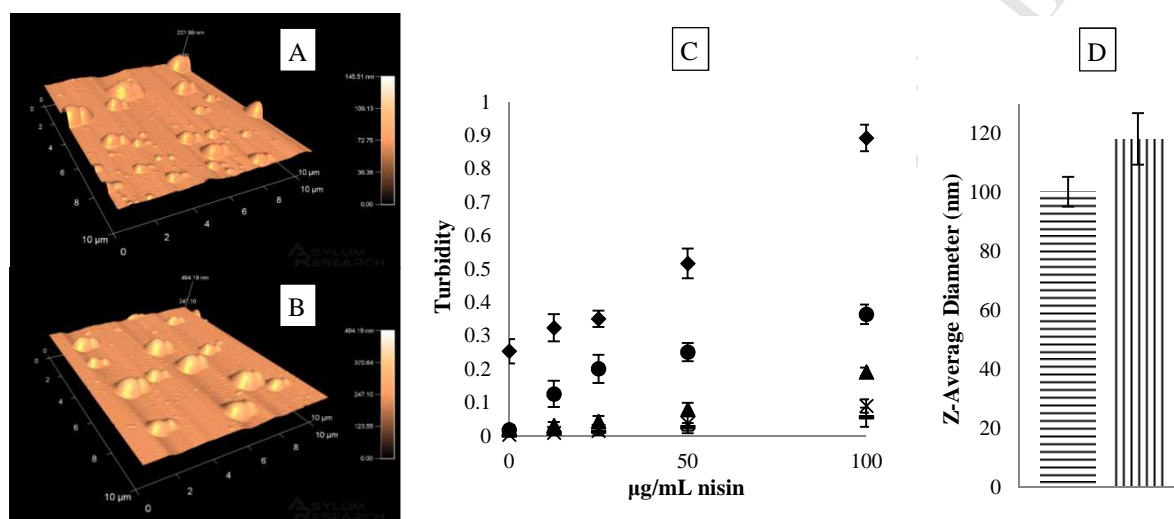
Surfactant	MIC ( $\mu\text{g/mL}$ )
No surfactant	1.81 ( $\pm 0.11$ )
0.3 millimoles/L bile salts	0.05 ( $\pm 0.01$ )
8 millimoles/L Tween <sup>®</sup> 80	0.008 ( $\pm 0.001$ )
0.2 millimoles/L Triton <sup>™</sup> X-100	0.008 ( $\pm 0.001$ )



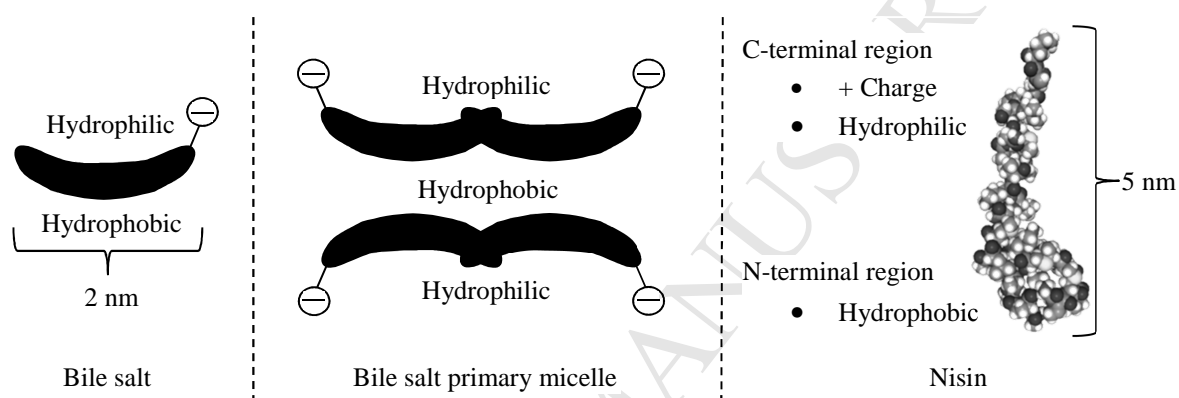
**Fig. 1.** RP-HPLC chromatograms of (A) undigested nisin and (B) the products of nisin digestion without bile (—) and with bile included (----). Regions where nisin fragments and intact nisin eluted are highlighted. The effect of including or excluding bile from a digestion with respect to the amount of nisin fragments 1-12, 1-29 and 1-20 produced is highlighted.



**Fig. 2.** Mass spectrometry analysis and well diffusion activity assay (inserts) of nisin fragments produced by the digestion of nisin. Analysis of intact nisin from the products of the control digestion is included for comparison in which 3354.70 Da correlates with the predicted molecular mass of intact nisin (3355.12 Da) (Chan et al., 1996) and 1678.14 Da correlates with the molecular mass of doubly charged intact nisin.



**Fig. 3.** Effect of nisin-surfactant interaction on particle size as examined by Atomic Force Microscopy AFM (A and B), Turbidity (C) and Dynamic Light Scattering (DLS) (D). AFM: AFM of bile (A) and AFM of bile with nisin (B). Turbidity (C): ◆ 10 millimoles/L bile salts, ● 0.3 millimoles/L bile salts, ▲ 0.2 millimoles/L Triton™ X-100, × 8 millimoles/L Tween® 80 and — No surfactant ( $\pm$  SD,  $n = 4$ ). DLS (D): ≡ 10 millimoles/L bile salts and ||| 10 millimoles/L bile salts with 100  $\mu\text{g/mL}$  nisin ( $\pm$  SD,  $n = 4$ ). Difference in particle size determined by DLS (D) is significant ( $p = 0.0123$ ,  $n = 4$ ).



**Fig. 4.** Schematics of a bile salt, a bile salt primary micelle and a nisin peptide. A bile salt molecule is 2 nm long (Small, 1971) and a nisin peptide is 5 nm long (Sahl, 1994). Drawings of a bile salt and primary micelle are based on those of Small (1968). Bile salt primary micelles can consist of 2 to 10 bile salts (Li et al., 2009). The image of nisin was produced using Protein Database entry 1WCO (Hsu et al., 2004) in conjunction with the NGL Viewer (Rose & Hildebrand, 2015).



*In vitro* digestion of nisin under physiological gastrointestinal conditions.

Six nisin fragments produced, four of which are bioactive.

Bile forms a complex with nisin.

Bile alters the relative amounts of the nisin fragments produced by digestion.